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IN VIVO ANTITUMOR ACTIVITY OF HERBIMYCIN A, A TYROSINE KINASE INHIBITOR, TARGETED AGAINST BCR/ABL ONCOPROTEIN IN MICE BEARING BCR/ABL-TRANSFECTED CELLS

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Abstract—Herbimycin A, a benzoquinoid ansamycin antibiotic, has been shown to reverse the oncogenic phenotype of $p60^{v\text{-}src}$ transformed cells because of the inhibition of src protein tyrosine kinase. We previously demonstrated that herbimycin A displayed antitumor activity on the *in vitro* growth of Philadelphia chromosome-positive leukemia cells and BCR/ABL-transfected murine hematopoietic FDC-P2 cells through the inhibition of BCR/ABL protein tyrosine kinase. In this study, the transformed FDC-P2 cells were demonstrated to be tumorigenic in syngeneic DBA/2 mice. The intraperitoneal (i.p.) injection of the transformed tumor cells into DBA/2 mice induced infiltrations of abdominal organs, and then all of the mice died within time periods proportional to the cell numbers of inoculation. In mice that received an i.p. inoculation with greater than 1×10^5 cells, *in vivo* administration of herbimycin A by i.p. injection inhibited tumor formation and significantly prolonged survival time, and further, in mice inoculated with 1×10^4 cells, herbimycin A completely suppressed the *in vivo* growth of transformant FDC-P2 cells and brought about a complete remission. The present study revealed the *in vivo* efficacy of herbimycin A in mice bearing BCR/ABL-transfected cells.

Key words: Herbimycin A, BCR/ABL oncoprotein, protein tyrosine kinase, tyrosine kinase inhibitor, *in vivo* antitumor activity.

Introduction

The *bcr/abl* oncogene is the product of the Philadelphia chromosome (Ph^1), resulting from the reciprocal translocation between chromosomes 9 and 22 which are found in Ph^1-positive leukemias which mainly constitutes more than 90% of chronic myelogenous leukemia (CML) and approximately 20% of adult acute lymphocytic leukemia (ALL) [1–5]. The *bcr/abl* fusion proteins generating from the hybrid *bcr/abl* genes, $P210^{bcr/abl}$ and $P190^{bcr/abl}$ exhibit the deregulated tyrosine kinase activity of ABL and oncogenic activity *in vitro* and *in vivo* [6–9].

Recent progress in the study of the *bcr/abl* gene and its product in Ph^1-positive leukemia offers an opportunity for developing several strategies for specific therapy targeted against BCR/ABL [10–14]. Herbimycin A, an inhibitor of protein tyrosine kinase (PTK) that we isolated on the basis of its ability to cause rat kidney cells transformed by v-*src* to revert to normal morphology with a loss of PTK activity of the transforming protein of $p60^{src}$[15], was shown to display a relatively selective antitumor activity for transformed cells by oncogenes coding PTK[16]. In fact, we demonstrated that herbimycin A displayed a preferential antitumor activity on the *in vitro* growth of Ph^1-positive cells by reducing BCR/ABL PTK [12]. In order to determine whether or not herbimycin A may offer a new therapeutic potential for Ph^1-positive leukemia, we have investigated the antitumor activity of *in vivo* administration of herbimycin A in syngeneic DBA/2 mice inoculated with BCR/ABL oncoprotein-transformed murine FDC-P2 cells. Here, we report the *in vivo* efficacy of herbimycin A on mice bearing BCR/ABL oncoprotein-associated tumors.

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Materials and Methods

Murine hematopoietic cell lines

The murine IL-3 dependent hematopoietic cell line FDC-P2 [17] was used in this study. As control cells, P815,

mastcytoma cell line, and P388, lymphoma cell line, were used.

Transfection of retroviral vector expressing $P210^{bcr/abl}$ into IL-3-dependent FDC-P2 cells

Five million FDC-P2 cells were cultured by the addition of a mixture of 30 µl Lipofectin (Gibco, Berlin, Germany) and 4 µg of pGD210 retroviral vectors expressing $P210^{bcr/abl}$ oncoprotein [8] for 16 h in serum-free Opti-MEM (Gibco) in the presence of recombinant murine IL-3 (rmIL-3) which was prepared from COS-1 cells transfected with p*SV2neo* containing mIL-3 cDNA. The cells were selected in a RPMI-1640 medium to which 10% fetal bovine serum (FBS) and 600 µg/ml geneticin (G418 sulfate; Gibco) were added in the presence of rmIL-3 for 10 days, and were then further selected in a medium lacking rmIL-3 for IL-3-independent growth. In the absence of mIL-3, parental or mock pZIPNeoSV(X)-transfected FDC-P2 cells died within 72 h, but G418-resistant $pGD210^{bcr/abl}$-transfected cells were able to grow autonomously in a medium which lacked mIL-3. As previously described in detail [12], transformed FDC-P2 cells expressed *bcr/abl* and *neo* mRNAs and the $P210^{bcr/abl}$ oncoprotein.

In vitro *growth testing for herbimycin A*

The assay of cellular proliferation was carried out by using the colorimeric MTT method. The cells (2×10^4/well) were seeded in triplicate in 100 µl of 10% FBS-added RPMI 1640 with or without herbimycin A, and cultured for 3–5 days in a humidified atmosphere with 5% CO_2 at 37°C. After the addition of 10 µl of a 5 mg/ml stock solution of MTT (3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) (Sigma Co. Ltd., Munich, FRG) into each well for the last 4 h, formazan crystals were solibilized in 100 µl of isopropanol–0.04 N HCl, and then the plates were measured on a MTP-100 microplate reader (Corona Electric, Tokyo, Japan) using a test wavelength of 570 nm with a reference wavelength of 630 nm.

Western blotting and immune complex kinase assay of BCR/ABL

Transformant FDC-P2 cells were cultured for 16 h in the presence or absence of 0.2 µg/ml of herbimycin A, where cell viabilities of both groups were more than 90%. The cells were lysed in the presence of kinase and phosphatase inhibitors, as previously described by Longo [18]. The lysate was clarified by centrifugation at 4°C, and 20 µg of proteins in the supernatant were separated by SDS-PAGE in reducing conditions (100 mM dithiothreitol). Proteins were blotted onto a polyvinylidene difluoride (Millipore) membrane. The BCR/ABL oncoprotein was identified using a monoclonal anti-ABL protein (Ab-3, Oncogene Science, Mineola, NY).

The immune complex kinase assay was carried out by using a modified method of that described by Kurzrock *et al.* [19, 20]. In brief, the cells (1×10^7) were harvested, washed with phosphate-buffered saline (PBS), and then lysed at 4°C by sonication in 50 mM Tris–HCl pH 7.5, containing 0.15 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF. The diluted sample was treated with mouse monoclonal antibody against c-*abl*, Ab-3 (Oncogene Science), for 1 h at room temperature and was incubated overnight at 4°C. Immune complexes with anti-ABL antibody were precipitated with mouse anti-IgG antibody-coated protein A (Zymed Laboratories, San Francisco, CA), and immunoprecipitates were then reacted with 5 µCi of (γ – ^{32}P) ATP for 10 min on ice. Next, immune complexes were analyzed on 7.5% SDS-polyacrylamide gels, followed by autoradiography with an intensifying screen.

Animals

Seven-week-old female DBA/2 mice were supplied by Oriental Kobo Co. Ltd (Tokyo, Japan).

Examination of tumor cells and tissues

Tissues were processed for histological examination and for Southern blot analysis. High molecular DNAs derived from tissues and tumors were digested with restriction enzymes, electrophoresed through 0.8% agarose gel, and then transferred to nylon membranes. The hybridization with a human 3′-*bcr* genomic DNA probe (*bcr*-1: Oncogene Science) was carried out by the method previously described [21].

Administration of herbimycin A

Herbimycin A was isolated as described previously [15], and was dissolved in phosphate-buffered saline containing 1% (V/V) of Tween-20. Various concentrations of herbimycin A were then administrated by i.p. injections daily from days 2–6 and from day 12 to day 16 after either transformed FDC-P2 cells, P518 cells or P388 cells at cell numbers of 1×10^4–1×10^6 were inoculated intraperitoneally into syngeneic DBA/2 mice on the first day.

Expression of results

Unless otherwise indicated, mean values ± one S.D. for measurements from triplicate culture have been presented. Bars in figures indicate one S.D. The statistical analysis was carried out using a Student's *t*-test, and results were interpreted to be significantly different when $p < 0.001$.

Results

In vitro *antitumor effect of herbimycin A on BCR/ABL-transformed murine cells*

We investigated the *in vitro* effect of herbimycin A against $P210^{bcr/abl}$ oncoprotein-associated autonomous growth of FDC-P2 cells transformed by a transfection of the *bcr/abl* gene. Herbimycin A showed no significant inhibition of the growth of parental FDC-P2 cells in the presence of the culture supernatant of WEHI cells containing mIL-3, but did display an inhibitory effect on the growth of transformant FDC-P2 cells at doses showing no toxicity on murine P815 cells and P388 cells (Fig. 1).

Effect of herbimycin A on BCR/ABL oncoprotein and tyrosine kinase

We previously reported that the antitumor activity of herbimycin A on Ph^1-positive leukemia cells seems to be associated with an inhibition on the activity of BCR/ABL PTK in spite of having no effect on the expression of BCR/ABL oncoprotein [12]. Similarly, the immune complex kinase assay of BCR/ABL revealed that herbimycin A dramatically suppressed

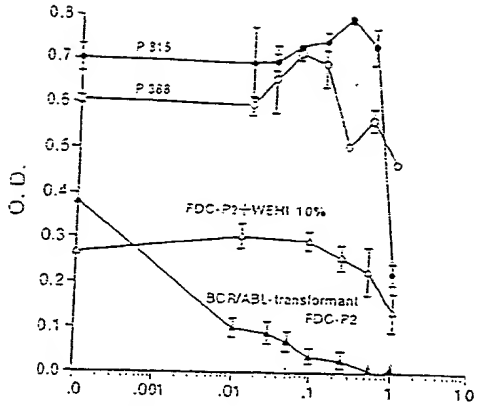

Fig. 1. Effects of herbimycin A on the *in vitro* growth of parental FDC-P2 cells, BCR/ABL-transfected FDC-P2 cells and syngeneic murine hematopoietic malignant cells. Effects of herbimycin A on IL-3-dependent growth of parental FDC-P2 cells in the presence of the culture supernatant obtained from WEHI cells and autonomous growth of transformant FDC-P2 cells by a transfection with BCR/ABL were assayed by MTT proliferation assay after culturing for 72 h. In P815 and P388 cells, MTT proliferation assay was carried out after the 72 h and 5 day cultures, respectively. The growth of P815 cells and P388 cells was not significantly inhibited at low doses of herbimycin A showing an inhibitory effect on the growth of transformant FDC-P2 cells in the 72 h and 5 day cultures. The results of 5 day cultures of P815 and P388 cells are shown.

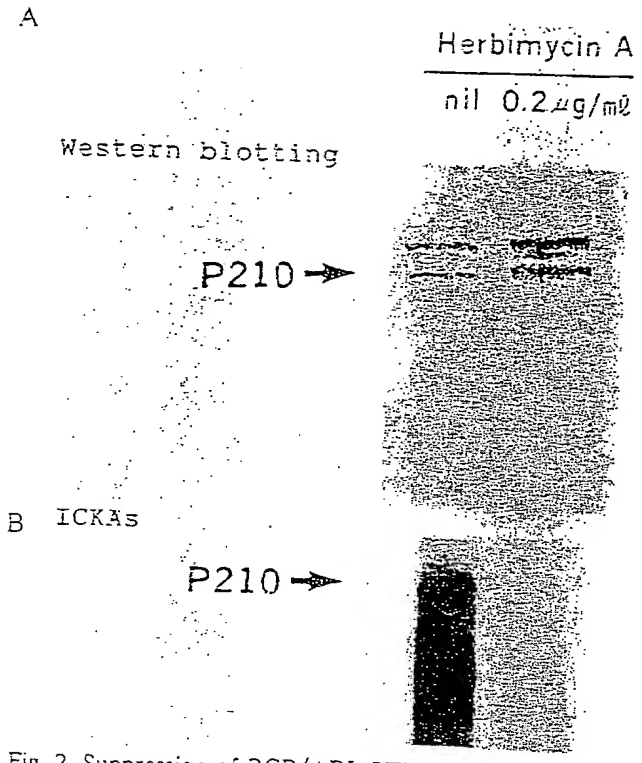

Fig. 2. Suppression of BCR/ABL PTK activity by herbimycin. (A) For the western blotting of BCR/ABL oncoprotein, transformant FDC-P2 cells were cultured for 16 h in the presence or absence of 0.2 μg/ml of herbimycin A, and then cellular protein was isolated. Twenty micrograms of protein were subjected to western blotting. (B) For the immune complex kinase assay (ICKAs), transformant FDC-P2 cells were treated with or without 0.2 μg/ml of herbimycin for 16 h. After this the cell extracts were immunoprecipitated with anti-c-*abl* monoclonal antibody. The resulting immune complexes were analyzed for their autophosphorylation activities.

the autophosphorylation activity of P210$^{bcr/abl}$ tyrosine kinase in BCR/ABL-transformed FDC-P2 cells, while it did not affect the amount of BCR/ABL oncoprotein (Fig. 2).

Mice bearing tumor cells transfected with BCR/ABL

Transformant FDC-P2 cells were transplantable by either i.p. or i.v. injection into syngeneic DBA/2 mice, whereas parental FDC-P2 cells were not transplanted. Mice inoculated with transformant FDC-P2 cells by i.p. injections exhibited a gradual increase of abdominal tumors and died within 5–11 weeks post-inoculation. Postmortem examinations revealed that tumor cells formed masses on the abdominal walls and infiltrated mainly into mesentric and retroperitoneal lymph nodes (Fig. 3(A)). The invasions were observed in the liver, spleen, pancreas and peritoneum (Fig. 3(A)), but were not noted in other organs including the lungs and kidneys. The presence of the *bcr/abl* gene in tumors was confirmed by Southern blot analysis with a human *bcr* probe (Fig. 3(B)).

In vivo efficacy of herbimycin A in mice bearing BCR/ABL oncoprotein-associated tumors

The study regarding determination of lethal dose in DBA/2 mice has revealed that LD_{50} of a single i.p. injection of herbimycin A is approximately 20 mg/kg body weight. The intraperitoneal injection of herbimycin daily for 5 days at less than 2.0 mg/kg body weight was well tolerated with no obvious toxicity. A marked reduction in subsequent tumor formation and a prolongation in the survival rates were evident following herbimycin treatment at doses of 1.0–2.0 mg/kg body weight in mice inoculated with greater than 1×10^5 of transformant cells (Fig. 4(A)). Furthermore, the i.p. administration with herbimycin A at 2.0 mg/kg body weight completely protected against tumor formation and brought about cures in all mice inoculated with 1×10^4 cells, whereas the i.p. administration at 1.0 mg/kg protected against tumor formation in two out of five mice (Fig. 4(B)). In contrast, P815 cells and P388 cells, which were not sensitive to herbimycin A *in vitro*, were transplantable into syngeneic DBA/2 mice, and all mice inoculated i.p. with amounts of greater than 1×10^4

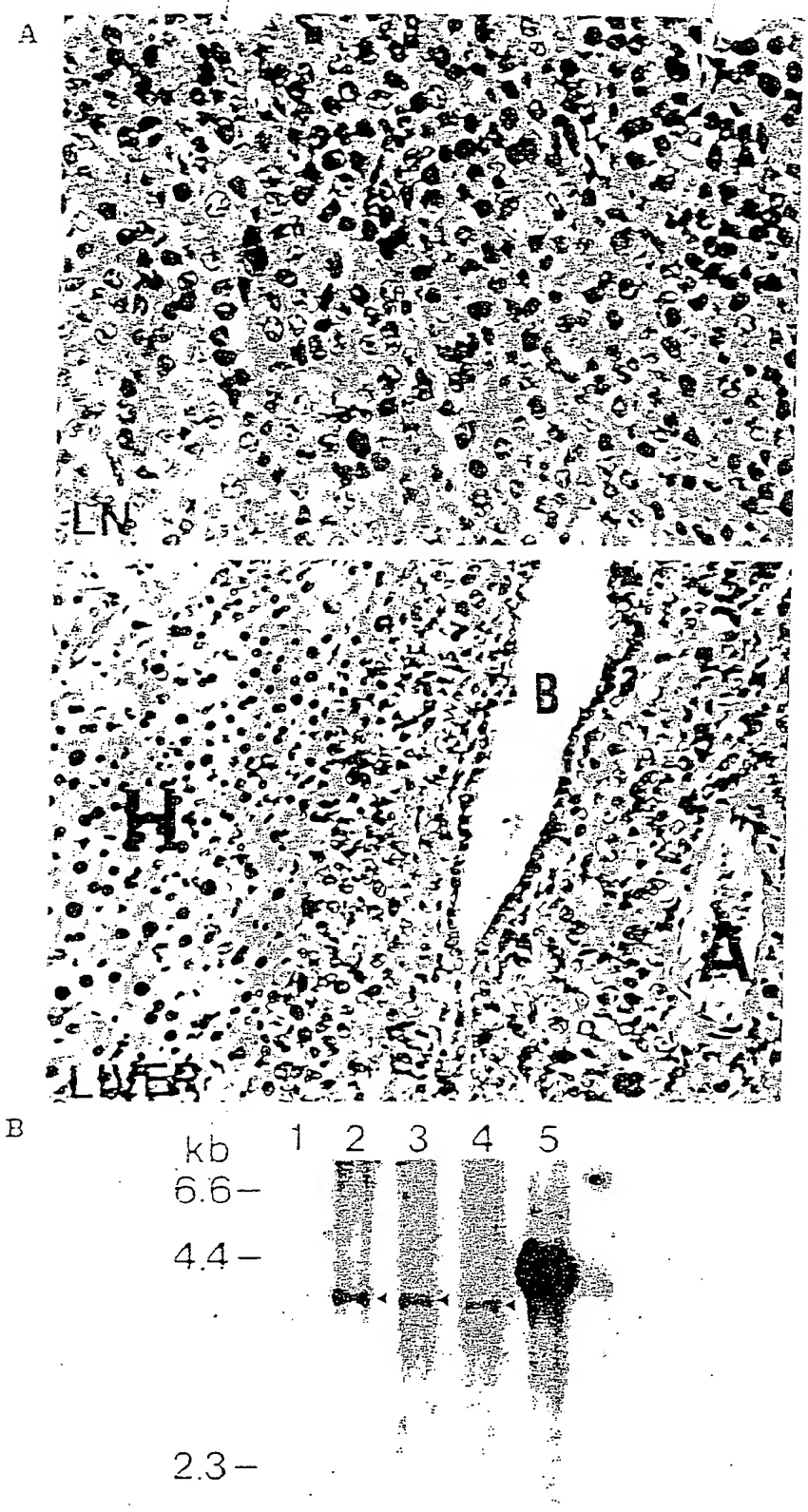

Fig. 3. Histological examination of tumors. (A) Pathological findings of the lymph nodes and the liver. Micrograph of the retroperitoneal lymph node (LN) showing the replacement by tumor cells. Small polygonal cells with prominent nuclei are evident (HE, ×350). Micrograph of the liver: tumor cells are observed throughout the portal tract (HE, ×200). B, bile duct; A, hepatic artery; H, normal hepatocytes. (B) Southern blot analysis revealed the presence of the *bcr/abl* gene. High-molecular weight DNAs isolated from tissues were digested with *Hind*III and were subjected to the Southern blot using 3′ M-*bcr* genomic DNA probe. Lane 1, parental FDC-P2 cells; lane 2, transformed FDC-P2 cells; lane 3, lymph nodes; lane 4, liver; lane 5, human placenta DNA. Arrows indicate the signals derived from *bcr/abl* gene.

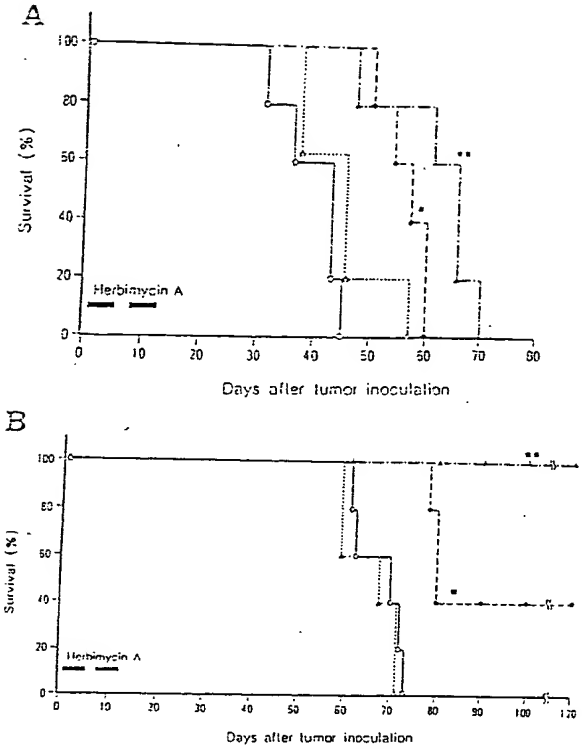

Fig. 4. Therapeutic effects of herbimycin A on DBA/2 mice inoculated with BCR/ABL-transformed FDC-P2 cells. Five DBA/2 mice per group were administered i.p. with herbimycin A (0.1 mg/kg body weight, Δ----Δ; 1.0 mg/kg body weight, ●----●; 2.0 mg/kg body weight, ▲----▲) on days 1–5 and 8–12 after i.p. inoculation of 1×10^6 tumor cells (A) or 1×10^4 tumor cells (B). Control groups (O-O) were administered with vehicle only. ■, ■■: significant, $p < 0.001$ (Student's *t*-test).

cells died within 12 weeks. In this case, herbimycin A administration had no effect on their survival rates.

Discussion

PTK activity has been demonstrated to be associated with stimulatory effects of many growth factors and with transforming oncogenes such as *src*, *abl*, *ros*, *fps*, *yes*, and *fes*. These findings indicate that a variety of PTK may play a critical role in the proliferation and differentiation of cells [22] as well as in some malignant transformations of cells, where Ph^1-positive leukemia is representative in human malignancies. Until now, several tyrosine kinase inhibitors including genistein, erbstain and lavendustin have been developed on the basis of inhibition of EGF-associated tyrosine kinase. In contrast to these inhibitors of receptor-type tyrosine kinase, herbimycin A, which was isolated from the cultured filtrate of *Streptomyces* sp. MH237-CF-8, was capable of causing a reversion of the transformed Rous sarcoma virus-infected rat kidney cell to normal morphology at modest concentrations via reduction of $p60^{src}$ PTK activity [15]. Furthermore, we previously demonstrated that herbimycin A preferentially suppressed Ph^1-positive leukemia cell growth and BCR/ABL oncoprotein-associated transformed murine cells via the inhibition of BCR/ABL PTK activity [12].

In this study, we evaluated the *in vivo* efficacy of herbimycin A in a murine model bearing BCR/ABL oncoprotein-expressing transformed cells. The transformed FDC-P2 cells by a transfection of BCR/ABL grew autonomously *in vitro* and were tumorigenic in syngeneic DBA/2 mice, in which the i.p. inoculation of transformant FDC-P2 cells showed the induction of massive swelling of abdominal lymph nodes and the infiltration of abdominal organs and brought about fatal outcomes in mice. In this murine model bearing the BCR/ABL-associated tumors, the i.p. administration of herbimycin A at concentrations of less than 2 mg/kg body weight for 5 days consecutively for 2 weeks significantly prolonged the survival times of the mice when they were inoculated with more than 1×10^5 tumor cells and, furthermore, completely protected against *in vivo* tumor formation in mice that received 1×10^4 cells with no overt side effects. Similarly, Honma has previously described the *in vivo* antitumor effect of herbimycin A in the murine model inoculated with highly v-*abl*-expressing murine myeloid cell lines, Cl cells [23]. Recently, it has been shown that herbimycin A also is a potent cytotoxin against malignant human tumors with primitive neural features, and the tumorigenicity in nude mice of these sensitive cell lines can be markedly reduced by systemic or topical administration of herbimycin A without any apparent toxicity to the whole animal [24].

More recently, erbstain and tyrphostins, derivative synthetics of erbstain, provided an important insight into future therapeutic developments against various types of cancers with amplified ErbB-1 (EGF-R) and ErbB-2 (Neu) through inhibiting PTK activity [25–27]. Furthermore, it has been noted that PTK blockers from the tyrphostin family can discriminate between normal ABL and transforming ABL proteins [28]. In addition to these studies, our development and study of herbimycin A, and further development of chemical modifications might provide an important insight into future therapeutic development according to a new strategy of *bcr/abl* oncoprotein-targeted therapy against Ph^1-positive leukemia.

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